ELSEVIER

Journal of Chromatography A, 891 (2000) 93–98

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Discoloration of ceramic hydroxyapatite used for protein chromatography

Scot R. Shepard*, Charmaine Brickman-Stone, Jeffrey L. Schrimsher, George Koch

Purification Process Development Department, Covance Biotechnology Services, Inc., 3000 Weston Parkway, Cary, NC 27513, USA

Received 16 February 2000; received in revised form 25 May 2000; accepted 25 May 2000

Abstract

Hydroxyapatite chromatography was used to purify a recombinant human protein at preparative (400 g) scale. The hydroxyapatite column became progressively discolored as the number of chromatographic cycles increased. Elemental analysis showed that Mn, Fe, Al, Cd, Ba, Cr and Sn were found in used, discolored hydroxyapatite but were below the detection limit (1 ppm) in new hydroxyapatite. Metal ions were not removed from the discolored hydroxyapatite by regeneration with 0.5 *M* sodium phosphate followed by 0.5 *M* sodium hydroxide. Chromatographic performance was not affected by the accumulation of metal ions for at least 8 cycles on the preparative column (media volume 56 l) and for at least 12 cycles on the laboratory-scale column (media volume 3.7 ml). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Hydroxyapatite; Stationary phases, LC; Preparative chromatography; Elemental analysis; Optical emission spectrometry; Proteins; Metal cations

1. Introduction

Tiselius et al. first introduced protein purification by hydroxyapatite liquid chromatography in 1956 [1]. Most matrices used for adsorptive liquid chromatography are comprised of an insoluble matrix that is derivatized with a specific ligand. In the case of hydroxyapatite, however, an insoluble hydroxylated calcium phosphate [$Ca_{10}(PO_4)_6(OH)_2$] forms both the matrix and ligand. Functional groups consist of pairs of positively charged calcium ions, referred to as C-sites, and clusters of negatively charged phosphate groups, referred to as P-sites [2] arranged in a crystalline structure. In general, positively charged amino groups on proteins interact with negatively charged P-sites and protein carboxyl groups interact by coordination complexation to C-sites. Thus, hydroxyapatite provides a charge-based, mixed-mode functionality derived both from the composition and spatial arrangement of constituent atoms. The simultaneous presence of both C- and P-sites provides a unique selectivity for the purification of large amphoteric molecules such as proteins.

The recent commercial introduction of mechanically stable, macroporous ceramic hydroxyapatite particles has increased the popularity of preparative-scale hydroxyapatite protein chromatography. We have used the multifunctional selectivity of hydroxyapatite to separate an intact recombinant human protein from proteolyzed forms of the same molecule. The separation was performed on a 56-l column loaded with 400 g of protein per chromatographic

*Corresponding author. Tel.: +1-919-3885-673; fax: +1-919-6780-366.

E-mail address: scot.shepard@covance.com (S.R. Shepard).

0021-9673/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(00)00626-9

cycle. In our application, we have observed a discoloration of the hydroxyapatite column that increased with the number of times the column was used. It is important to understand how many times a large preparative chromatography column can be reproducibly recycled, since the frequent replacement of resin can have a considerable impact on manufacturing costs. This paper reports the identification of chemical differences between new and used hydroxyapatite and the effect of these changes on the chromatographic separation.

2. Experimental

2.1. Chemicals

Ceramic hydroxyapatite was purchased from Bio-Rad (Hercules, CA, USA). Sodium phosphate and sodium hydroxide were USP grade from J.T. Baker (Phillipsburg, NJ, USA). Water used for buffer preparation was water-for-injection (WFI) grade. Briefly, WFI is the specific grade of water required by regulatory agencies for injection into humans. Consequently, WFI is routinely used for the production of parenteral grade biopharmaceuticals. Information and specifications for WFI are available in the United States Pharmacopeia [3]. Process buffers and protein solutions were prepared and/or stored in stainless steel tanks.

2.2. Elemental analysis

Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to measure the amount of metallic elements in solid and liquid samples. The analysis was performed in a Perkin-Elmer Optima 3000XL ICP spectrometer. Briefly, samples digested in nitric acid were aerosolized into argon plasma. Elements excited by the argon plasma were then identified, upon return to the ground state, by their characteristic emission spectra. Emission intensity was then converted to elemental concentration by comparison to a standard curve. Samples were analyzed under steady state conditions and 1 ppm was used as the sensitivity limit. Elemental analysis was performed by Quantitative Technologies, Inc. (Whitehouse, NJ, USA).

2.3. Total carbon, hydrogen and nitrogen analysis

Carbon, hydrogen and nitrogen (CHN) were determined using a Perkin-Elmer CHN elemental analyzer. Briefly, samples were oxidized by complete combustion in an oxygen environment. Product gases were then separated under steady state conditions and quantified as a function of thermal conductivity. These analyses were performed by Quantitative Technologies, Inc.

2.4. Preparative-scale hydroxyapatite chromatography

2.4.1. Chromatography conditions and sampling of used resin

Hydroxyapatite was used as the third chromatography step in a process designed to purify a recombinant human protein from *Pichia pastoris* fermentation. The columns preceding the hydroxyapatite step included both cation- and anion-exchange resins. The purity of the protein at this point was 62% ($n=8$). The hydroxyapatite column was pre-equilibrated with five column volumes of 500 m*M* sodium phosphate, pH 7.0, and then equilibrated with 10 m*M* sodium phosphate, pH 7.0. Protein (400 g) was loaded onto the column (35 cm length×45 cm diameter=56 l) at 4 l/min. After loading, unbound proteins were removed by washing the column with equilibration buffer. Protein was eluted with a five-column volume continuous gradient from 10 m*M* sodium phosphate, pH 7.0, to 74 m*M* sodium phosphate, pH 7.0. The column was then regenerated with five column volumes of 500 m*M* sodium phosphate, pH 7.0 followed by cleaning with five column volumes of 0.5 *M* NaOH. The column was stored in 0.1 *M* NaOH between cycles. All chromatography was performed at 18–22°C. After eight chromatography cycles the top approximately 6 cm of the hydroxyapatite bed was a dark, rust brown color. At this point, a sample was taken from the top of the packed bed and the used medium was analyzed by ICP-OES and CHN analysis. Unused

hydroxyapatite, taken directly from a new container, was analyzed in parallel as a control.

2.4.2. Protein recovery and purity

The recovery and mass purity of the eluate fraction was calculated for each of the eight chromatography cycles performed on the preparative hydroxyapatite column. Total protein recovery was calculated by dividing the mass of protein eluted from the column by the mass loaded onto the column multiplied by 100. The mass purity of the hydroxyapatite elution fraction was determined by analytical reversed-phase high-performance liquid chromatography (RP-HPLC). Mass purity was calculated as the ratio of the area under the product peak to the total area under all peaks as detected by absorbance at 215 nm.

2.5. Hydroxyapatite cycling studies

Small-scale hydroxyapatite cycling studies were performed using the separation conditions described above. The column (19 cm length×0.5 cm diameter=3.7 ml) was loaded with 27 mg of protein. The separation was run at 2 ml/min and was controlled with an ÄKTA liquid chromatography system (Pharmacia, Uppsala, Sweden). The column effluent was monitored at 280 nm. The performance of the new column (cycle 1) was compared to the used column (cycle 12). Protein recovery into the elution fraction was calculated as above.

Elution peak asymmetry, plate count and selectivity factor (relative to the unbound peak) were calculated for cycle 1 and cycle 12 for the small-scale cycling studies. Equations and theory for these calculations have been reviewed previously [4].

The resolution of the elution peak, relative to the column unbound fraction, was calculated for the new and used column. The resolution (R_s) for adsorptive chromatography using linear gradient elution is approximated by:

$$R_s \propto \sqrt{\frac{L}{gV_0H}} \quad (1)$$

where L is the column length, g is the gradient slope, V_0 is the void volume and H is the plate height [4,5].

3. Results and discussion

3.1. Elemental analysis

Elemental analysis by ICP-OES was used to measure the concentration of 64 elements in both new and used (eight cycles) hydroxyapatite. Table 1 shows the results for each element with a detectable concentration greater than 10^{-6}% (w/w) (1 ppm). The ICP-OES data show that new hydroxyapatite was composed primarily of Ca and P (O and H are not detectable by this method). Traces of Na, Mg, Si, K, Sr, Zn, B, and Ni were also detected in the sample of new hydroxyapatite. All elements found in new hydroxyapatite were also found in used hydroxyapatite. However, Mn, Fe, Al, Cd, Ba, Cr and Sn were found in used hydroxyapatite but were below the detection limit (1 ppm) in new hydroxyapatite. The amount of Mn increased by approximately 100 000 times and all elements present in new hydroxyapatite in trace quantities increased significantly in used hydroxyapaptite except for Zn, B, and Ni. The large increase in Na may be explained by the

Table 1
Results of ICP-OES elemental analysis for both new and used hydroxyapatite

Element	New hydroxyapatite (%, w/w)	Used hydroxyapatite (%, w/w)	Difference[a]
Ca	51.9	45.8	−6.0
P	24.7	23.4	−1.3
Na	$7.0\cdot10^{-6}$	3.8	3.8
Mn	–[b]	0.1	NA
Fe	–[b]	$3.6\cdot10^{-4}$	NA
Mg	$6.0\cdot10^{-6}$	$2.1\cdot10^{-4}$	$2.1\cdot10^{-4}$
Al	–[b]	$4.0\cdot10^{-5}$	NA
Si	$1.4\cdot10^{-5}$	$3.4\cdot10^{-5}$	$2.0\cdot10^{-5}$
K	$1.2\cdot10^{-5}$	$2.8\cdot10^{-5}$	$1.6\cdot10^{-5}$
Cd	–[b]	$2.3\cdot10^{-5}$	NA
Ba	–[b]	$2.0\cdot10^{-5}$	NA
Sr	$7.5\cdot10^{-6}$	$2.0\cdot10^{-5}$	$1.2\cdot10^{-5}$
Cr	–[b]	$1.4\cdot10^{-5}$	NA
Sn	–[b]	$6.5\cdot10^{-6}$	NA
Zn	$7.0\cdot10^{-6}$	$6.5\cdot10^{-6}$	$-5.0\cdot10^{-7}$
B	$2.0\cdot10^{-6}$	$4.0\cdot10^{-6}$	$2.0\cdot10^{-6}$
Ni	$3.0\cdot10^{-6}$	$3.0\cdot10^{-6}$	0.0

[a] Difference only calculated for elements detected in new hydroxyapatite.
[b] Below detection limit of $1\cdot10^{-6}$% (w/w) (1 ppm).

fact that the hydroxyapatite sample sent for ICP-OES was a slurry in 10 m*M* sodium phosphate.

3.2. Total carbon, hydrogen and nitrogen analysis

Total CHN analysis was performed on new and used (eight cycles) hydroxyapatite. New hydroxyapatite contained 0.25% C, 0.33% H and <0.05% N. The total nitrogen and hydrogen content of new and used media were very similar; indicating that used hydroxyapatite was not contaminated with residual protein. The total carbon content of used hydroxyapatite (0.25%) was higher than new hydroxyapatite (0.17%) which indicates an increase in the total carbon content of used media after purification of approximately 3.2 kg of recombinant protein.

3.3. Performance of the preparative hydroxyapatite column

The total protein recovery for the preparative hydroxyapatite column ranged from 60 to 80% with an average of 66% and a standard deviation of 6 ($n=8$). The mass purity of the elution fraction ranged from 73 to 86% with an average of 81% and a standard deviation of 5 ($n=8$). The preparative column was cycled only eight times prior to removal of the discolored resin at the top of the column. The results demonstrate a random variation that is within the expected range for a large-scale operation.

3.4. Small-scale hydroxyapatite column cycling

Fig. 1 shows the chromatographic profiles of the hydroxyapatite separation for cycle 1 and cycle 12 (*x*-axis offset 7% for clarity). Comparison of the profiles shown in Fig. 1 reveals little to no change in the chromatographic separation. Consequently, calculation of peak asymmetry, plates/m, resolution and selectivity gave nearly identical results for cycles 1 and 12. The values for peak asymmetry were 2.80 and 2.95, the number of plates (N) were 845 m^{-1} and 846 m^{-1}, resolution were 1.8 and 1.8, and selectivity were 5.42 and 5.43 for cycle 1 and cycle 12, respectively. Protein recovery into the elution fraction, 69.3% for cycle 1 and 68.9% for cycle 12, also remained essentially unchanged. Column performance data suggest little to no change in the

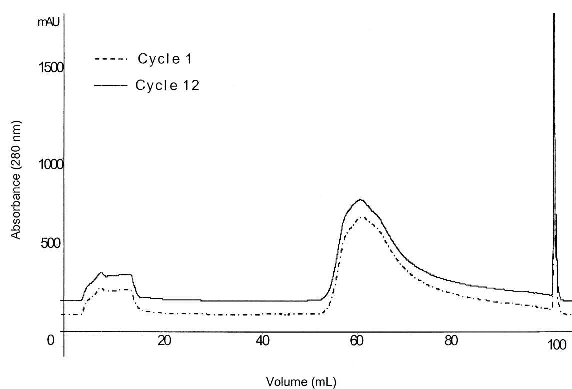

Fig. 1. Comparison of the hydroxyapatite chromatographic profiles for cycles 1 and 12 of the small-scale cycling experiment. For each sample 27 mg of protein was injected.

chromatographic separation over 12 cycles; however, visual inspection of the column after 12 cycles showed that 4–6 cm of the 19 cm column was stained a dark, rust brown color. The total protein recovery data for the small-scale cycling experiments are in good agreement with the recovery data for the preparative column suggesting a valid scale-down model.

Peak asymmetry measurements are sensitive to column voids, heterogeneous packing, uneven flow distribution and/or column clogging. The plates/m calculation can detect zone broadening, a change in particle size, a change in column void volume, and/or a change in solute diffusion coefficients. The lack of large detectable differences in peak asymmetry and plates/m calculations indicates that the integrity of the packed column did not change over the course of 12 chromatography cycles.

Equations describing column resolution and selectivity were also applied to the chromatographic profiles. The same column and elution gradient were used for cycles 1 and 12, so resolution is then sensitive to effects of void volume (V_0) and the factors contributing to plate count (N). The resolution did not change over the course of 12 cycles. The selectivity factor is calculated from retention volume, which is a function of the type of charged group, the number of charges, and the pore structure of the matrix as it relates to availability of adsorptive sites [4]. The selectivity factor calculation did not change over the course of 12 runs. The lack of

detectable differences in resolution and selectivity indicate that the chemical changes detected in the hydroxyapatite by ICP-OES did not affect the separation.

3.5. Potential sources of metal ions

The data in Table 2 show the ICP-OES results for the protein solution before and after hydroxyapatite chromatography. The average volume before chromatography was 146 l and after chromatography averaged 271 l. Inspection of the data does not clearly identify the protein load solution as the only source of the metal ions that accumulated on the hydroxyapatite column. For example, Mn is not detectable in the protein solution before or after hydroxyapatite chromatography; however, used hydroxyapatite was 0.1% (w/w) Mn. Therefore, some of the metal impurities must be introduced into the process from other sources. Potential sources of contamination include: metals added as process chemicals, impurities present in process chemicals, impurities found in process water, or as ions that have leached from processing equipment.

Sodium phosphate and sodium chloride salts used for chromatography are the likely sources of most of the P and Na detected in the protein solution. Fermentation media was supplemented with cupric sulfate, sodium iodide, manganese sulfate, sodium molybdynate, boric acid, cobalt chloride, zinc chloride, ferrous sulfate and zinc sulfate. These metals, which were intentionally added to the fermentation media as micronutrients, could be carried to the hydroxyapatite step. Cation-exchange purification of the fermentation broth was the first step in the process. Cations could adsorb to the negatively charged media and co-elute with the protein. Flow-through purification on an anion-exchange resin was the second step in the process and it is possible that cations would pass through the column along with the unabsorbed protein. Hydroxyapatite, the third step in the process, could then concentrate various metals present in the protein solution.

Phosphate salts used for buffer preparation, which are known to have high levels of contaminating metals, were potential sources of metal impurities. For each hydroxyapatite cycle in the preparative application the column was exposed to a volume and concentration of phosphate buffer equivalent to more than 80 kg of solid sodium phosphate. Salts of various metals, for example the salts used to add metals to the fermentation media, are also known to contain relatively high levels of other metals as impurities [6]. Metals can also leach from stainless steel into WFI-grade water and process buffers and protein solutions that are prepared and/or stored in stainless steel tanks.

Table 2
ICP-OES analysis of the protein solution before and after hydroxyapatite chromatography

Element	Before chromatography (%, w/w)	After chromatography (%, w/w)
Ca	$4.6\cdot10^{-5}$	$8.0\cdot10^{-6}$
P	0.11	0.20
Na	0.55	0.26
Mn	–[a]	–[a]
Fe	$8.0\cdot10^{-6}$	–[a]
Mg	$3.4\cdot10^{-4}$	$6.7\cdot10^{-5}$
Al	$1.0\cdot10^{-6}$	$1.0\cdot10^{-5}$
Si	$3.1\cdot10^{-5}$	$3.4\cdot10^{-5}$
K	$7.0\cdot10^{-6}$	$2.0\cdot10^{-6}$
Cd	–[a]	–[a]
Ba	–[a]	–[a]
Sr	–[a]	–[a]
Cr	–[a]	–[a]
Sn	–[a]	–[a]
Zn	–[a]	–[a]
B	$1.0\cdot10^{-5}$	$1.0\cdot10^{-6}$
Ni	–[a]	–[a]

[a] Below detection limit of $1\cdot10^{-6}$% (w/w) (1 ppm).

4. Conclusions

Elemental analysis by ICP-OES demonstrated that hydroxyapatite used for protein chromatography became modified by the accumulation of metal ions.

The data showed that Mn, Fe, and Mg (in that order) were the most concentrated of the contaminating metal ions detected. Previous work reported that Fe induced changes in the crystal structure of hydroxyapatite used in surgical implants [7] and MnO_4 has been detected in hydroxyapatite contaminated with Mn [8]. Nutritional studies have shown that patients using hydroxyapatite as a calcium supplement exhibited reduced Fe uptake when Fe and Ca were supplemented at the same time [9]. A

recent report discusses the use of hydroxyapatite modified with Zn or Fe for purification of histidine tagged proteins [10]. Thus, the binding of metal ions by hydroxyapatite has previously been described in the biomaterials, nutrition and protein chromatography literature. The data in the present report are consistent with these previous findings and furthers the literature by demonstrating that small amounts of various metals, added to the process or present as process impurities, can bind to and accumulate on hydroxyapatite columns.

The performance of hydroxyapatite protein chromatography was consistent in the reported application despite the accumulation of metals on the media. The column used for preparative chromatography was consistent for at least eight cycles and the column used for small-scale cycling studies was consistent for at least 12 cycles. The chemical composition changes detected in discolored hydroxyapatite did not affect the protein recovery, peak asymmetry, column plate count, chromatographic resolution or selectivity of the hydroxyapatite separation to a degree that was detectable by the analyses performed. However, the effect of hydroxyapatite modification by metals may vary from application to application, with the amount of protein loaded on the column, with the amount and type of impurities present in the process, and with the number of times a particular column is used.

References

[1] A. Tiselius, S. Hjerten, O. Levin, Arch. Biochem. Biophys. 132 (1956) 65.

[2] P. Gagnon, in: Purification Tools for Monoclonal Antibodies, Validated Biosystems, Tucson, AZ, 1996, p. 87.

[3] The United States Pharmacopeia, Vol. 23, The United States Pharmacopeial Convention, Rockville, MD, 1995, p. 1635.

[4] G. Sofer, L. Hagel, in: Handbook of Process Chromatography – A Guide to Optimization, Scale-up, and Validation, Academic Press, New York, 1997, p. 256.

[5] S. Yamamoto, K. Nakanishi, R. Matsuno, in: Ion-Exchange Chromatography of Proteins, Marcel Dekker, New York, 1988, p. 258.

[6] P. Gagnon, Summer 1999 Validated Biosystems Quarterly Resource Guide to Downstream Processing, Validated Biosystems, Tucson, AZ, 1999.

[7] J. van der Meulen, H.K. Koerten, J. Biomed. Mater. Res. 28 (1994) 1455.

[8] L. Yubao, C.P. Klein, X. Zhang, K. de Groot, Biomaterials 13 (1993) 969.

[9] B. Dawson-Hughes, F.H. Seligson, V.A. Huges, Am. J. Clin. Nutr. 44 (1986) 83.

[10] T. Nordstrom, A. Senkas, S. Eriksson, N. Pontynen, E. Nordstrom, C. Lindqvist, J. Biotechnol. 69 (1999) 125.